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ARTICLE

Synergistic dynamics of light, photoperiod and chemical stimulants influences biomass and lipid productivity in *Chlorella singularis* (UUIND5) for biodiesel production

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Abstract Microalgae have emerged as a potential alternative for the production of many useful compounds like protein, carbohydrate and lipid. Lipid-rich microalgae are important and rich source for alternative energy production. In order to commercially utilize microalgae for energy production, the lipid productivity should be enhanced. Keeping in view the above-mentioned potentials of microalgae, in the present study, we have attempted to display the role of chemical stimulants and light in the growth and lipid production of the microalgae *Chlorella singularis* (UUIND5). During the present investigations, effect of varying photoperiods and different types of lights and chemical stimulants, viz. $CaCl_2$ and kinetin on growth rate and lipid production, was studied. The maximum growth rate recorded was 166 ± 0.3 mg/L/d, when 0.80 g/l $CaCl_2$ and 0.5 mg/l kinetin were added to Bold's basal medium. *C. singularis* was then cultivated in this medium for 14 days under sunlight +LED (10-h sunlight + 14-h LED light) at photoperiod 24-h light/0-h dark. The maximum lipid yield 30.2% of dry wt. was obtained under sunlight +LED. Further, the gas chromatography analysis also showed the presence of fatty acid methyl esters (FAME). FAMEs profile was analyzed according to ASTM D6751 specification. Thus, it was concluded that sunlight +LED at 24-h light/0-h dark (100 µmol photons m^{-2} s^{-1}) photoperiod with $CaCl_2$ and kinetin is an effective strategy to boost lipid productivity in *C. singularis* (UUIND5).

Keywords *Chlorella singularis* · Microalgae · Light · $CaCl_2$ · Kinetin

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Introduction

Rapid rise in fossil fuel demand throughout the world is increasing fossil fuel depletion and carbon emissions leading to global climate change. This has intensified the discovery of the alternative fuels. Among the different options available for alternative energy production, microalgae are currently attracting wide interests. This is because by photoautotrophic mechanism microalgae convert CO_2 into biomass, lipid (fatty acid) and protein. The total lipid content in microalgae varies from 10 to 70% of dry algae biomass from species to species and has 20–40 times more productivity than oil crops [1–3]. For the growth of microalgae, light is an important factor. Excessive intensity may cause photo-oxidation, and low intensity decreases the growth [4]. Various artificial lights are used by the researchers to increase the production capacity. Development of light-emitting diode (LED) light presents an enormous potential for improving microalgae growth. The light duration itself is an important factor for microalgae [5]. For industrial-scale production of microalgae, the ratio between the cost of energy and the biomass productions is an important factor for lowering per unit cost of biodiesel. For this, preference is given to outdoor cultivation where light energy comes directly from the sun [6]. But sunlight has certain drawbacks such as changing day and night cycles in summer and winter [7, 8]. UV radiations are another growth-limiting factor for

microalgae. UV radiations are typically divided into three categories: UV-A (lower energy) wavelength 320–400 nm, UV-B (higher energy) wavelength 280–320 nm and UV-C (higher energy) wavelength 254–280 nm [9]. Out of three categories of UV light, UV-B and UV-C have serious effects on biologically important macromolecules, i.e., lipid, proteins and nucleic acids of plant and animals because these cellular components can absorb UV-B and UV-C radiation directly [9, 10].

In view of this literature, the present study has various specific objectives, which are listed as follows: The first is to isolate a novel strain of microalgae from freshwater river and propose an optimized photoperiod for growth. In addition, the investigation is carried out to determine the effect of different lights (LED, CFL, sunlight and UV). Second is to study the effect and standardized the concentration of $CaCl_2$ and kinetin for increasing the growth of microalgae. Finally, the potential of biomass to produced lipid and biodiesel is explored.

Materials and methods

Isolation of microalgae strain

Microalgae samples were collected from the freshwater Tons River, Dehradun, Uttarakhand, India. Pure culture was isolated by serial dilution and then streaking the samples on to 1% Bold's basal medium (BBM) agar plate. Bold's basal medium (BBM) was prepared according to the composition given by Guarnieri et al. [11]. Isolation of single strain was done according to Tale et al. [12]. For microalgae strain identification, DNA was isolated and 18S rRNA was amplified using the forward primer ITS1-TCCGTAGGTGAACCTGCGG and reverse primer ITS4-TCCTCCGCTTATTGATATGC. Hundred publically available *Chlorella* sp. sequences were downloaded from NCBI. Isolated strain (*Chlorella singularis* UUIND5) was identified based on their 18S rRNA sequence by constructing phylogenetic trees using MEGA 6 software [13].

Variable photoperiod and light experiments

To determine which photoperiod would be suitable targets for biomass applications, growth trials were conducted on all photoperiod (6-h light/18-h dark, 12-h light/12-h dark, 16-h light/8-h dark and 24-h light/0-h dark) under visible light (CFL/LED).

Visible light treatment experiments

Microalgae were grown at 25 °C and 16-h light/8-h dark photoperiod for 4 days. After 4 days, microalgae strain was subjected to visible light at 24-h light/0-h dark photoperiod for 10-days treatment. The visible light treatment was given under compact fluorescent light (CFL) (100 µmol photons m^{-2} s^{-1}) and LED (blue wavelength 450–495 nm, 100 µmol photons m^{-2} s^{-1}). Sunlight+LED light was also used during the study. The 24-h light/0-h dark photoperiod was completed as 10-h sunlight + 14-h LED.

UV treatment experiments

After the 4 days of initial growth period, the microalgae strain was subjected to ultraviolet radiation with UV lamps UV-B (280–320 nm) irradiation with a density of 5.7 (Wm^{-2}) for period for 1 h per day for 10 days. To complete 24-h light/0-h dark photoperiod after the UV treatment, 23-h LED (100 µmol photons m^{-2} s^{-1}) light was provided.

Chemical stimulants experiments

To provide further insights into the performance of microalgae isolate, effect of $CaCl_2$ and kinetin at different concentration (0.10–1 g/l BBM to $CaCl_2$ and kinetin 0.1–0.9 mg/l BBM) growth trials was also conducted.

Estimation of biomass productivity and lipid accumulation

The novel microalgae strain growth was measured using a UV–Vis spectrophotometer (UNICO model 2100 spectrophotometer). Samples were taken every 2 days for 14 days.

$$\text{CDW}\ (\text{g/L}) = 0.274\,\text{OD686 nm} + 0.002$$

Biomass productivity (mg/L/d) was calculated according to equation.

$$P = \frac{(\text{CDW}_x - \text{CDW}_1)}{t_x - t_1}$$

where CDW_x and CDW_1 are the cell dry weight at time t_x and t_1 (the time recorded after lag phase) [14].

The lipid accumulation in *Chlorella singularis (UUIND5)* was measured using Nile red method.

Total lipids extraction

Lipids were extracted from fresh microalgal biomass using a modified method of Bligh and Dyer [16]. Chloroform–methanol (1:2, v/v) was used for the extraction of total lipids. The total lipids obtained were measured gravimetrically, and percentage of lipid and lipid productivity (mg/L/d) were calculated by the following equations:

$$\text{Lipid yield}\ \% = \text{Lipid content}\ (\text{g})/\text{Dry algae biomass}\ (\text{g})$$

$$\text{Lipid productivity} = \text{Biomass productivity} \times \text{Lipid yield}\,(\%)/100$$

Physicochemical property analysis of lipid

For triacylglycerols (TAGs) detection, lipid sample (5 µl) was spotted on silica gel plate and TAGs were visualized according to Patel et al. method [17]. Acid value, iodine value, and saponification value were analyzed according to AOCS methods [18].

Biodiesel production—acid-catalyzed transesterification

The total extracted lipids were transesterified into fatty acid methyl esters (FAMEs) by methanolic sulfuric acid (6%) [15]. The FAMEs were analyzed using gas chromatography–mass spectroscopy (GC–MS; Agilent technologies, USA). One microliter of sample was injected and process completed according to Patel et al. method [17].

Fuel properties of algal biodiesel

The biodiesel obtained from algal cultures further analyzed for the physicochemical properties such as iodine value, saponification value, specific gravity, acid value, cetane number, high heating value, long-chain saturation factor and specific gravity was determined according to ASTM D-6571 specifications. Fire and flash point were determined by Pensky–Martens closed cup tester.

Estimation of pigments, protein and carbohydrate content

The growth performances under different lights, photoperiods and chemical stimulants were studied by chlorophyll estimation. Total chlorophyll was estimated according to the protocol described by Lichtenthaler [19]. Total protein isolation and estimation were done by a method given by Slocombe et al. [20]. Total carbohydrate content was estimated by Kumar et al. method [15].

Statistics

The statistical analysis was carried out by analyzing the triplicate ($n = 3$) results for each culture. These results have been reported as mean ± SD. The data were further validated by one-way ANOVA using Graph Pad Prism software (version 6.0f) with $p < 0.05$.

Results

Isolation and identification of microalgae strain

The isolated microalgae was identified as *Chlorella singularis* UUIND5 (GenBank accession number: KY745895). The phylogenetic tree was constructed based on neighbor-joining analysis of 18S rRNA sequence revealed that *Chlorella* sp. (Fig. 1).

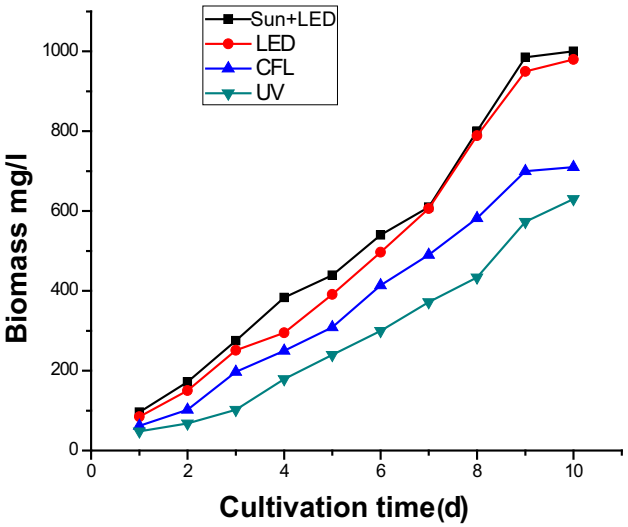

Fig. 2 Growth of *Chlorella singularis* (UUIND5) grown under different lights for a period of 10 days. The data are mean ± SD for triplicate ($n = 3$) results ($p < 0.05$)

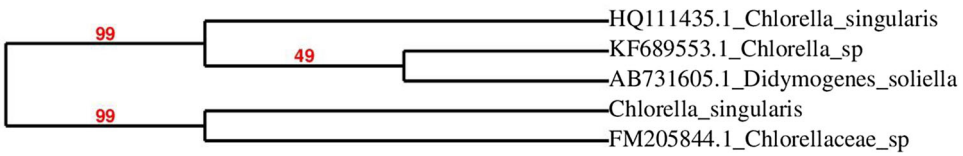

Fig. 1 Phylogenetic tree showing the relationships among partial 18S rRNA sequences of isolate *Chlorella singularis* (UUIND5)

Table 1 The *Chlorella singularis* (UUIND5) lipid properties on 14th day

Parameter	Sunlight + LED	LED	CFL	UV
Total lipid content	30.2%	29.3%	25.5%	19%
Iodine value	90 ± 0.03	86 ± 0.01	89 ± 0.01	120 ± 0.01
Saponification value (mg of KOH/g)	136 ± 0.01	123 ± 0.02	142 ± 0.01	158 ± 0.02
Acid value (mg of KOH/g)	142 ± 0.03	136 ± 0.02	148 ± 0.02	156 ± 0.01

The data are mean ± SD for triplicate ($n = 3$) results ($p < 0.05$)

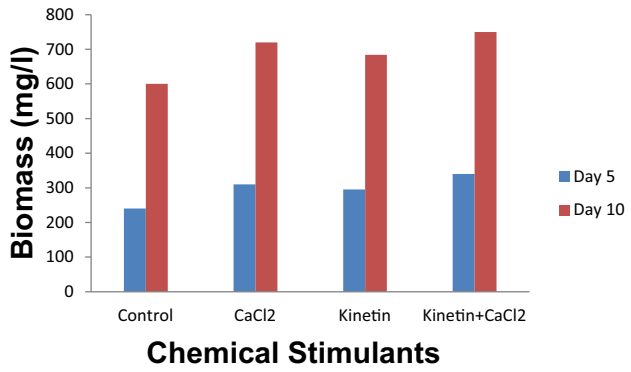

Fig. 3 Effect of chemical stimulants on biomass productivity. The data are mean ± SD for triplicate ($n = 3$) results ($p < 0.05$)

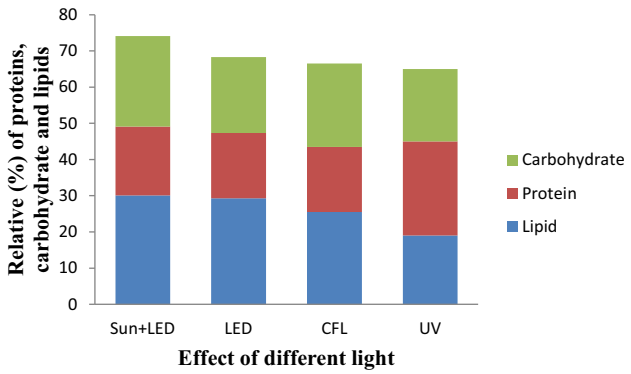

Fig. 4 Effect of different lights on protein, carbohydrate and lipid contents of *Chlorella singularis* (UUIND5). The data are mean ± SD for triplicate ($n = 3$) results ($p < 0.05$)

Biomass productivity and lipid productivity analysis

In this study, we have observed that maximum biomass productivity was attained in 24-h light/0-h > photoperiod followed by 16-h light/8-h dark > 12-h light/12-h dark > 6-h light/18-h dark photoperiod. So for this study, we have choosed 24-h light/0-h dark photoperiod (100 µmol photons m^{-2} s^{-1}). The growth profile of *Chlorella singularis* (UUIND5) under LED light was maximum and low growth under UV light (Fig. 2).

Light had an impact on total biomass and lipid productivity in *Chlorella singularis* (UUIND5) culture (Table 1). Sunlight + LED light consistently yielded a one fold increase in biomass productivity as compared to CFL light (Fig. 3).

Effect on pigments, protein and carbohydrates

In this study, the effect of different lights on pigments, protein and carbohydrates was observed (Fig. 4 and Table 2). The experimental results showed that UV-B irradiance caused the reduction in the contents of chlorophyll a, b and chlorophyll (a + b) and carotenoid.

Physicochemical property analysis of fatty acid and biodiesel

TLC confirmed presence of TAGs in the total extracted lipids. The overall comparison of different light effects displayed that light not only could lead to different fatty acid yields, but also affect the fatty acid profile to a

Table 2 Effect of different lights on *Chlorella singularis* (UUIND5) pigments on 10th day

Different lights	Chl *a** (µg/ml)	Chl *b*** (µg/ml)	Car*** (µg/ml)	Chl *a* + Chl *b*	Chl *a*/Chl *b*	Car/Chl *a* + Chl *b*
Sun + LED	9.61 ± 0.05	2.60 ± 0.01	3.58 ± 0.03	12.22 ± 0.05	3.69 ± 0.03	0.29 ± 0.03
LED	5.81 ± 0.02	0.37 ± 0.01	1.47 ± 0.02	5.43 ± 0.03	15.44 ± 0.01	0.45 ± 0.02
CFL	5.61 ± 0.02	0.32 ± 0.04	1.84 ± 0.02	5.93 ± 0.04	17.52 ± 0.01	0.31 ± 0.01
UV	1.71 ± 0.03	0.56 ± 0.03	0.93 ± 0.01	2.28 ± 0.02	3.017 ± 0.02	0.40 ± 0.01

The data are mean ± SD for triplicate ($n = 3$) results ($p < 0.05$)

*Chlorophyll a, **Chlorophyll b, ***Cartenoids

large extent (Fig. 5). Palmitic acid (C16:0), heptadecanoic acid (C17:0), 7,10-hexadecadienoic acid methyl ester (C16:2), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), 9,12-octadecadienoic acid (C19:2) were obtained in large amounts under sunlight+ LED light. Small amounts (0.18%) of pentadecanoic acid (C15:0) was present in LED-treated biomass.

Discussion

For industrial-scale production of microalgae to reduce the cost of energy, we recommend sunlight + LED light. Our finding supported the finding of various researchers who reported that alone LED lights found unsuitable for microalgae growth if used without additional light sources [7, 21, 22]. In contrast, the UV light inhibition growth produced significantly less biomass than cultures treated with other lights (Fig. 2). The maximum biomass productivity of 166 ± 0.3 mg/L/d was attained under sunlight + LED followed by LED > CFL > UV, respectively. Wang et al. [23] reported that microalgae biomass produced under LED light is economical as grams of biomass per liter per dollar. LED lights consume less power and yield high productivity at less cost [24]. Katsuda et al. [25] showed blue LED light enhancing the growth of *Haematococcus pluvialis*. Posten [26] reported that LED has the ability to distribute light uniformly in the bioreactor. Toe et al. [7] reported that 24-h light/0-h dark (100 µmol photons m^{-2} s^{-1}) photoperiod of LED light gave the maximum productivity. Low light intensity (50–100 µmol photons m^{-2} s^{-1}) increasing the photoperiod from 12-h light/12-h dark to 24-h light/0-h dark increases the specific growth and division rates in the microalgae [27].

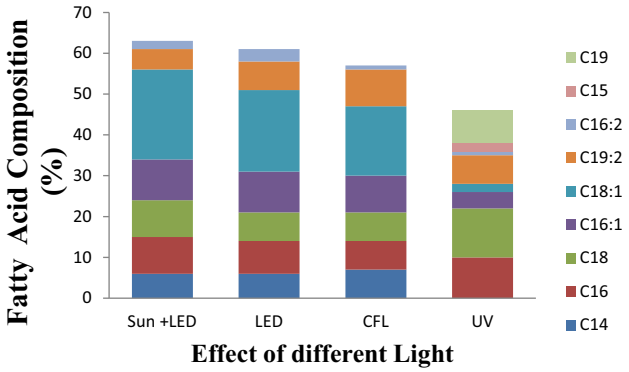

Fig. 5 Effects of different lights on fatty acid methyl ester profile of *Chlorella singularis* (UUIND5). The data are mean ± SD for triplicate ($n = 3$) results ($p < 0.05$)

$CaCl_2$ and kinetin increased the biomass, lipid, pigments and carbohydrates productivity in microalgae as compared to control (Fig. 3). Xu et al. [28] reported that $CaCl_2$ can help to avoid the stress effects by increasing biomass, chlorophyll content, antioxidant enzymes, proline content in plants. Calcium plays an important role in many defense mechanisms [29]. Sabi et al. [30] reported that kinetin improved the uptake of nitrogen, phosphorus and potassium contents in plant. Sadak et al. [31] also reported positive effects on biomass, pigments, total carbohydrate, protein and total phenolic contents of plants.

It was reported that UV irradiance reduced the chlorophyll and carotenoid contents in algal biomass [32]. Our study revealed an increase in protein level of up to 12% under UV-B light as compared to LED light. Hartmann [33] also reported increase in protein level in UV-treated

Table 3 *Chlorella singularis* (UUIND5) biodiesel fuel properties

Properties	Units	Different lights				ASTM D6751	Commercial biodiesel
		Sun + LED	LED	CFL	UV		
Saponification value	(mg KOH)	114.63	110.16	96.82	80.26	–	
Iodine value	(g I_2/100 g)	35.42	39	36	24	–	130
Specific gravity	(kg^{-1})	0.691	0.723	0.700	.639	–	–
Acid value mg	KOH g^{-1}	2.4	2.7	2.2	5.2	0.8	0.50
Flash point	°C	42	40	39	33	93.0 min	35
Fire point	°C	50	52	48	42	–	–
Cetane value		58.02	54.52	60.63	50.19	47 (min)	47
High heating value		40.20	46.12	42.06	39.3 2		
Long-chain saturation factor	(% wt)	15.7	10.2	10.6	16.6	–	–
Cold flow plugging property	°C	2.4	1.68	2.65	4.70	–	− 5

– No standard limit designated by ASTM D6751-02 biodiesel standards

algae. UV radiation-induced protein accumulation protects plants against UV radiation [32].

The UV-treated biomass mainly contained hexadecanoic acid (C16), 9-octadecenoic acid (C19), pentadecanoic acid (C15:0), less amount of 9,12-octadecadienoic acid (C19:2), 9-octadecenoic acid (C19), pentadecanoic acid (C15:0). Lipid content and PUFA decrease with increase in light intensity [34].

FAMEs obtained from microalgae have combustion properties similar to conventional diesel fuel. Important parameters of biodiesel are cetane number, iodine value, cloud, fire, flash point and oxidative stability which determine the quality of fuel, stability and performance are summarized in Table 3. Results of FAME parameters were similar to the findings of Kumar et al. [35].

From the results, we have concluded that maximum growth rate was reported at 24-h light/0-h dark photoperiod (100 µmol photons m^{-2} s^{-1}). High lipid productivity was observed under sunlight + LED-treated *Chlorella singularis* (UUIND5) cells. UV light-treated cells showed low chlorophyll, carotenoids, carbohydrates content and high protein content. FAMEs profile was analyzed according to ASTM D6751 specification. It was observed that all the properties fit within the standard limits. Sunlight + LED light at 24-h light/0-h dark photoperiod with $CaCl_2$ and kinetin is an effective strategy to boost biomass and lipid productivity in *Chlorella singularis* (UUIND5).

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